Zinc induces iron uptake and DMT1 expression in Caco-2cells via a PI3K/IRP2 dependent mechanism

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Running title: *Zinc induces intestinal iron absorption*

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Abstract:

The absorption of dietary iron is influenced by numerous dietary and physiological factors. We have previously demonstrated that zinc treatment of intestinal cells increases iron absorption via induction of the apical membrane iron transporter divalent metal iron transporter-1 (DMT1). To better understand the mechanisms of zinc-induced iron absorption we have studied the effect of zinc on iron uptake, iron transporter and iron regulatory protein (IRP 1 and 2) expression and the impact of thePI3K pathway in differentiated Caco-2 cells, an intestinal cell culture model. We found that zinc induces DMT1 protein and mRNA expression. Zinc-induced DMT1 expression and iron absorption were inhibited by siRNA silencing of DMT1. Further, zinc treatment led to increased abundance of IRP2 protein in cell lysates and in polysomal fractions, implying its binding to target mRNAs. Zinc treatment induced Akt phosphorylation, indicating the activation of the PI3K pathway. LY294002, a specific inhibitor of PI3K inhibited zinc-induced Akt phosphorylation, iron uptake, DMT1 and IRP2 expression. Further, LY294002 also decreased the basal level of DMT1 mRNA but not protein expression. siRNA silencing of IRP2 led to down regulation of both basal and zinc-induced DMT1 protein expression, implying possible involvement of post-transcriptional regulatory mechanisms. In agreement with these findings zinc treatment stabilized DMT1 mRNA levels in actinomycin-D treated cells. Based on these findings, we conclude that zinc-induced iron absorption involves elevation of DMT1 expression via stabilization of its mRNA, via a PI3K/IRP2-dependent mechanism.

Keywords: Zinc, Iron, interactions, IRP2, PI3K, DMT1, Akt, intestine, Caco-2 cells

Introduction:

Iron and zinc deficiencies coexist in populations subsisting on phytic acid-rich vegetarian diets [1] suggesting that fortification and/or supplementation with both iron and zinc should be considered to improve mineral status. However, some studies in humans, animals and cell culture models have indicated competitive interactions between iron and zinc at supplemental concentrations [2, 3]. Conversely, epidemiological studies found a strong positive association of serum zinc with haemoglobin levels [4, 5]. Furthermore, experimental zinc deficiency in rats leads to development of anaemia and reduced erythropoiesis [6]. Interestingly, separate delivery of iron and zinc leads to improved haematological responses compared to iron supplementation alone in children [7]. These observations suggest that though iron and zinc interact negatively when supplemented together, zinc may still have a positive impact on iron status. These effects may be mediated either by enhanced intestinal iron absorption or increased metabolic utilization of iron.

In the absence of obligatory excretory pathways, systemic iron homeostasis is primarily regulated by modulating intestinal absorption [8]. The divalent metal ion transporter-1 (DMT1), a proton-coupled metal ion transporter localized predominantly in duodenum [9], mediates the intestinal iron absorption. At the apical surface of the enterocyte, duodenal cytochrome B (DcytB) reduces ferric iron (Fe^{3+}) to ferrous (Fe^{2+}) [10] and facilitates its uptake via DMT1. Once absorbed, iron is either stored in ferritin or exported across the serosal membrane through a ferroportin (Fpn1)- hephaestin (HEPH)-mediated process [11, 12].

The cytosolic iron regulatory proteins 1 and 2 (IRP1 and IRP2), post-transcriptionally regulate expression of iron metabolic proteins by binding to iron responsive elements (IREs), stem loop structures, at the 5' or 3' untranslated regions

(UTRs) of target mRNA. Binding of IRPs to the 5' UTRs inhibits protein translation while binding to 3' UTRs stabilizes the mRNA and thereby increases protein expression [13]. IRP1 is a bifunctional protein, which requires disassembly of a 4Fe-4S cluster for activation. In contrast, IRP2 expression is inducible, depending on cellular iron levels, and levels are controlled by proteosomal degradation. Induction of IRP2 expression and/or activation of IRP1 during iron deficiency ensure increased iron absorption and mobilization from intestinal cells. Interestingly, in addition to iron status, a variety of stress conditions influence iron metabolism via IRP-dependent processes [14].

Previous studies in intestinal cells demonstrated that zinc treatment stimulates the iron uptake and transcellular transport by inducing DMT1 and Fpn1expression [15, 16]. Interestingly, zinc-induced Fpn1 expression has been shown to be mediated by MTF1 in mouse fibroblasts [17]. Though initial studies identified MREs in 5 promoter region of DMT1, latter studies ruled out such possibility [18] and therefore the mechanism of zinc induced DMT1 expression and iron absorption remained elusive.

Zinc has gained interest as a potent cell signalling mediator [19-23]. Zinc ions have been shown to activate numerous signalling pathways involving the receptor or non-receptor tyrosine kinases, Ras/mitogen-activated protein kinases(MAPKs) and the PI3K/Akt/p70 S6 kinase pathway [19-21, 23] and to inhibit the activity of protein tyrosine phosphatases [22]. Further, the zinc-induced metal regulatory transcription factor 1 (MTF1) phosphorylation and target gene expression has been reported to be mediated by PKC, PI3K and JNK dependent pathways [21].

The purpose of our current study was to gain a better understanding of the mechanisms of zinc-induced intestinal iron absorption. We have investigated the regulatory role of zinc-sensitive signal transduction pathways and have examined potential interactions with IRPs to regulate iron transporter expression. We

hypothesize that zinc-induced signalling events mediate an increase in DMT1 expression either directly or via IRP dependent mechanisms.

Materials and Methods:

Materials: IRP1, IRP2, Akt (P-Ser473 and pan-Akt) antibodies andLY294002were procured from Cell Signalling Technologies (MA, USA). DMT1 antibody was purchased from Santa Cruz biotechnology (CA, USA). β-actin antibody was from Abcam (Cambridge, MA, USA). The cell culture media components such as antibiotic-mycotic mix and trypsin are procured from Invitrogen (CA, USA). All other reagents were procured from Sigma Chemical Co. (Bangalore, India), unless specified.

Methods:

***Caco-2 cell culture*:** The human intestinal Caco-2 cell line was obtained from the American Type Culture Collection (HTB-37, ATCC, Rockville, MD, USA). Caco-2 cells were grown at 37°C in an atmosphere of 5 % CO_2 and 95 % humidity in Eagle's Minimum Essential Medium (MEM) supplemented with 10 % (v/v) heat inactivated foetal bovine serum (FBS), 1 % (v/v) penicillin/streptomycin (Invitrogen, Paisley, UK). For experiments, cells were seeded into 6-well plates and grown for 21 days to allow cells to fully differentiate. The cells were incubated in serum-free MEM for 12h and treated with $ZnSO_4$ (100 µmol/L) for the times indicated. LY294002 (25 µmol/L), where present, was added 30 min prior to the addition of zinc.

Iron uptake: The measurement of iron uptake by Caco-2 cells has been described previously [16]. Briefly, following zinc treatment, media was removed and replaced with 2mL of 2-(N-morpholino) ethane sulphonic acid (MES)-buffered salt solution (pH 6.5 containing: 140 mmol/L NaCl; 5 mmol/L KCl; 1 mmol/L Na_2HPO_4; 1 mmol/L $CaCl_2$; 0.5 mmol/L $MgCl_2$; 5 mmol/L glucose). Uptake was initiated by the addition of 10

μmol/L Fe^{2+} complexed with 1 mmol/L ascorbic acid (freshly prepared prior to the start of each experiment) and 37 kBq/mL $^{59}FeCl_3$. The reaction was terminated after 15 min, and cell monolayers were washed 3 times in ice-cold transport buffer containing a 10-fold excess of iron to remove non-specifically bound iron, solubilised overnight in 200 mM NaOH. The cell associated ^{59}Fe radioactivity was determined by counting in an Auto Gamma Counter (Wizard-2, Perkin Elmer).

Realtime PCR: Total RNA was isolated from cultured cells using TRIzol. Following cDNA synthesis, expression levels of DMT1 (+IRE and -IRE), IRP2 and β-2 microglobulin mRNA (used as a housekeeping gene) were analysed by real-time quantitative PCR using an ABI Prism 7500 FAST Sequence Detection System and a Power SYBR Green PCR master mix kit (New England Biosciences, UK). The primer sequences used for each gene are given in **Supplementary Table 1**. Quantitative measurements of target genes relative to the housekeeping gene were derived using the ΔCt method. Data are normalised to the untreated control group in each experiment and are presented as the mean ± S.E.M.

***Isolation of polysomes*:** IRP-1 and IRP-2 levels, after the incubations, were assessed in the polysomal fraction following treatments as described previously [24]. Briefly, cells were washed in ice-cold PBS and scraped into 3 mL of digitonin buffer (20 mmol/L Tris–Cl, pH 7.4; 250 mmol/L sucrose; 0.007% digitonin; 1× protease inhibitor cocktail). Cells were manually homogenized using 21 G and 26½ G needles and kept on ice for 15 min. The homogenate was subjected to sequential centrifugation at 1500 g (10 min), 10,000 g (10 min) and finally at 100,000g for 60 min. The pellets from the latter two steps enriched in polysomes were pooled and suspended in TX-100 buffer (20 mmol/L Tris–Cl, pH 7.4; 250 mmol/L sucrose; 1% TX-100; 5% protease inhibitor cocktail). The IRP levels in polysomal fraction were assessed by immunoblotting as described below.

Immunoblotting: Following treatments, the cell monolayers were washed (3X) with 10 mmol/L phosphate buffer saline pH 7.2 and lysed in RIPA buffer (Thermo Fisher) supplemented with protease inhibitor cocktail (1X), EDTA (1 mmol/L), Sodium orthovanadate (1 mmol/L), NaF (10 mmol/L). The protein content was estimated using micro-BCA kit method. Equal amount of protein (20-30 µg) was fractionated on 10% SDS-gels under reducing conditions and transblotted on to the PVDF membranes. The blots were blocked with 5% non-fat dry milk or BSA and probed with primary respective primary antibodies followed by respective commercially available secondary antibodies. The blots visualized using enhanced chemiluminescence detection kit (Bio-Rad, USA) and Hyperfilm ECL (Amersham Pharmacia Biotech) or images were acquired using G-box imaging system (Syngene, USA). The blots were re-probed with β-actin, used as a loading control. The images were quantified using Image-J software (NIH, USA) and normalized to respective loading controls.

Transient transfection of Caco-2 cells with siRNA*:* Caco-2 cells were seeded at a density of 1.0 X 10^5 cells/mL in complete media in 12-well plates and allowed to adhere for 10 days. The spent media was aspirated, and the cells were washed once with pre-warmed Dulbecco's Phosphate Buffered Saline (DPBS). Next, the cells were supplemented with OptiMEM (Life Technologies, Paisley, UK) containing 5% FBS without any antibiotics. One hour following the addition of OptiMEM, the Caco-2 cells were transfected with 10 nM of either DMT-siRNA (SLC11A2; M-007381; Dharmacon, CO, USA), IRP2-siRNA (S7498; Life Technologies, Paisley, UK) or a non-targeting scrambled siRNA (AM4635; Life Technologies, UK), using Lipofectamine 3000 (Life Technologies, Paisley, UK) according to manufacturer's protocol. 48 hours after transfection, the media was aspirated from each well, the cells were washed once with

pre-warmed DPBS, and supplemented with fresh OptiMEM containing 5% FBS for 72 hours.

mRNA stability: The Caco-2 cells were incubated either in the presence or absence of zinc (100 µmol/L) for a period of 4h, followed by addition of actinomycin-D (10 µg/mL). At 0, 2, and 4 h after addition of actinomycin D, cells were harvested; qPCR analysis of DMT1 was performed as described above. The mRNA remaining is expressed as a percentage of mRNA levels at t = 0 h.

Statistics: All data are expressed as mean ± SEM. Statistical analysis was carried out using SigmaPlot (version 12, Systat Software Inc. IL, USA). Student's unpaired t-test was used to compare differences between control and a single test group. One-way or Two-way ANOVA followed by Tukey's post-hoc test was used where appropriate to detect statistical differences ($P<0.05$) between multiple groups.

Results:

Our previous experiments demonstrated that zinc induces iron absorption in differentiated Caco-2 cells with a maximal effect at 100 µmol/L [16], hence all the experiments were performed with this zinc concentration.

Initial transport studies with Caco-2 cells grown on Transwell membranes showed a 30% increase in transepithelial iron transport following zinc treatment; this was blocked by the incubation withLY294002 (control, 100.0 ± 4.8%; + zinc, 128.3 ± 5.7%; zinc + LY294002, 93.4 ± 11.5%; $P<0.03$). To determine whether zinc was modulating iron transport at either the apical, or basal, or both surfaces of Caco-2 cells we measured DMT1 and Fpn1 expression. Zinc treatment for either 4 or 24 h significantly induced DMT1 (+IRE) mRNA ($P<0.01$) and DMT1 protein ($P<0.001$) expression (**Fig. 1 A and 1B**). DMT1 (-IRE) mRNA levels were low and in some cases

below the detection limits of our assay in both control and zinc-treated cells. Thus, we did not measure expression of this isoform. There was no significant effect on Fpn1 mRNA in these studies (control, 100.0 ± 16.0%; + zinc 4 h, 108.2 ± 12.0%; + zinc 24 h, 122.1 ± 19.7%). Consistent with the zinc-induced increase in DMT1 (+IRE) expression, zinc treatment also significantly increased (P<0.001) iron uptake by Caco-2 cells at the same time points (**Fig. 1C**).

To confirm that the effects of zinc on iron uptake were mediated through DMT1 we performed siRNA knockdown of DMT1. There was no significant difference in the values obtained for DMT1 protein and iron uptake between the un-transfected control group and cells transfected with scrambled siRNA (**Supplementary Fig. 1**). We therefore used the un-transfected control group for subsequent analysis. DMT1 protein levels were increased in un-transfected cells following exposure to zinc. Treatment with DMT1 siRNA led to significant down regulation (P<0.001) of DMT1 protein expression compared to control cells and levels remained significantly suppressed (P<0.001) in DMT1 siRNA cells treated with zinc (**Fig. 2A**). DMT1 silencing also significantly inhibited (P<0.001) the basal and zinc-induced iron uptake compared to controls (**Fig. 2B**).

Preliminary experiments revealed that zinc-induced iron uptake is inhibited byPI3K but not by JNK or PKC inhibitors (**Supplementary Fig. 2**). Zinc treatment increased phosphorylation of Akt (pSer-473) in a time-dependent manner without changes in total Akt protein expression, and this was blocked completely by LY294002, a potent inhibitor of PI3K (**Fig. 3A**). This prompted us to investigate the role of the PI3K pathway in more detail. Pre-treatment of Caco-2 cells with LY294002 significantly inhibited (P<0.01), zinc-induced iron uptake, DMT1 protein and mRNA expression (**Fig**

3B, C and D). Interestingly, LY294002 treatment alone also significantly inhibited ($p<0.001$) the DMT1 mRNA expression (**Fig. 3D**).

Incubation with zinc significantly increased ($P<0.001$) IRP2 protein expression in a time-dependent manner reaching maximum abundance between 0-2 h, and levels remained elevated in the presence of zinc thereafter. However, zinc had no effect on IRP1 expression (**Fig 4A**). Zinc concurrently induced IRP2 levels, but not IRP1, in polysomal fractions (this represents active IRPs bound to IREs in target mRNAs), as a function of time (**Fig 4B**). Zinc did not affect IRP2 mRNA levels over the time course of this study (**Fig 4C**). The effect of zinc on IRP2 protein expression was significantly inhibited ($P<0.001$) by LY294002 (**Fig. 4D**).

To determine whether zinc-induced changes in IRP2 expression mediated the regulation of DMT1 we carried out siRNA knockdown of IRP2. There was no significant difference in IRP2 protein levels between the un-transfected control group and cells transfected with scrambled siRNA (**Fig 5A lane 1 and 5**). Transfection with IRP2 siRNA, resulted in significant down regulation ($P<0.001$) of IRP2 protein expression compared to un-transfected control cells (**Fig. 5Aand B**). IRP2 silencing also significantly reduced ($P<0.001$) the basal DMT1 protein (**Fig. 5A and C**), but not DMT1 mRNA expression (**Fig. 5D**) compared to control cells. Furthermore, zinc failed to induce DMT1 protein (**Fig 5A and C**) or mRNA expression (**Fig.5D**) in IRP2-silenced cells compared to control. LY294002 further inhibited ($P<0.01$) the IRP2 and DMT1 expression in IRP2-silenced cells (**Fig 5A, B and C**), either in the presence or absence of zinc compared to respective controls.

To assess whether the zinc-IRP2 axis increased DMT1 (+IRE) mRNA stability we treated cells with the transcription inhibitor actinomycin D in the presence or absence of zinc. DMT1 (+IRE) mRNA decreased with time; however, the rate of decrease in

DMT1 mRNA levels was significantly lower (P<0.01) in cells treated with zinc + actinomycin-D compared to cells treated with actinomycin-D alone (**Fig. 6**).

Discussion:

Epidemiological studies have shown an association between zinc status, blood haemoglobin levels and iron status. A possible explanation lies in the observation that zinc increases intestinal iron absorption and DMT1 expression in a time-dependent manner [15, 16]. DMT1 is expressed in multiple isoforms that are differentiated primarily by the presence (+) or absence (-) of IRE at the 3'end of the transcribed mRNA [8]. In the present study we determined the expression of these isoforms. The expression of the DMT1 –IRE was below the limit of detection in our assay, whereas DMT1 (+IRE) was significantly increased by zinc.

Our previous work has shown that DMT1 is highly expressed at the apical membrane in Caco-2 cells. Using a neutralizing antibody to the transporter we demonstrated that it the primary transporter of non-haem iron in this intestinal cell line [25]. Further, knockout studies in mice demonstrate that DMT1 is essential for intestinal iron absorption [26]. Other putative transporters for iron are present in intestinal cells. For instance, multiple zinc transport proteins (Zips) have been reported to mediate iron uptake in cell culture models [27, 28], and some of these proteins are expressed in Caco-2 cells [29, 30]. However, the physiological relevance of these transporters in mediating iron absorption is unknown. Therefore, to confirm the specificity of DMT1 for iron transport in Caco-2 cells, we studied the impact of DMT1 silencing on zinc-induced iron uptake. Silencing of DMT1 led to significant inhibition of iron absorption in Caco-2 cells. These results are consistent with previous data from our group and from others and confirm that DMT1 is the predominant transporter of iron in

intestinal cells. Taken together with our mRNA and protein data, these findings indicate that zinc-induced increase iron absorption requires up regulation of DMT1 expression.

It is known that cellular iron homeostasis is primarily achieved by post-transcriptional regulation of iron metabolism proteins by IRPs [14, 13]. Binding of IRPs to IREs in 3'-UTR, which are present in DMT1 and TfR1 mRNA, stabilizes the target mRNAs, manifesting in higher expression of protein and thus increased iron absorption [13]. In contrast, enterocyte-specific ablation of IRPs (both 1 and 2) results in malabsorption of iron, leading to death in first four weeks [31]. Mice in these studies exhibited a marked reduction in duodenal DMT1 protein and DMT1 (+IRE) mRNA expression [31]. In the present study, DMT1 (+IRE) mRNA was maximally increased by zinc at 4 h and remained elevated until 24h while the protein expression continued to increase between 4-24 h, implying the possible involvement of post-transcriptional mechanisms mediated by IRPs. Thus, we studied the impact of zinc on IRP1 and 2 expression and activity. Zinc had no effect on IRP1 expression but markedly increased the IPR2 protein levels and its localization within polysomal fractions. Since the polysomal IRP levels represent the active IRE-bound pool [24], this suggests that zinc induces both the IRP2 levels and activity. Moreover, time course studies indicated that induction of IRP2 expression and its polysomal abundance occurs as early as 1 h following exposure to zinc, which is in advance of the observed increases in DMT1 mRNA and protein level. Therefore, we hypothesized that zinc-induced IRP2 expression might regulate DMT1 post-transcriptionally.

To further delineate the mechanism, we studied the impact of IRP2-silencingon zinc-induced DMT1 expression. IRP2-silencing significantly reduced basal DMT1 protein levels but not mRNA expression compared to cells transfected with scrambled siRNA. Interestingly, silencing of IRP2 also blocked the zinc-induced DMT1 protein and

(+IRE) mRNA expression, suggesting that activation of a zinc-IRP2 axis is required for stabilization of DMT1 mRNA. Treatment with actinomycin-D (a potent inhibitor of transcription) decreased DMT1 mRNA, this was attenuated significantly by zinc treatment, which further supports the notion that zinc induces DMT1 mRNA stability. The fact that zinc had no effect on IRP1 expression and activity, implies a critical mechanistic role of IRP2 in mediating zinc-induced DMT1 expression. In agreement with these results IRP2, but not IRP1, knockout mice exhibited marked reduction in macrophage TfR1 levels [32]. In these animals, IRP2 was demonstrated to be the predominant physiological regulator of iron homeostasis while IRP1 plays an important role during stress conditions such as increased oxygen tension [32]. In addition, increased TfR1 expression in Hela cells following insulin treatment has also been shown to be mediated by IRP2-dependent mRNA stabilization [33]. These results together suggest that zinc increases DMT1 expression in intestinal cells post-transcriptionally via IRP2-dependent DMT1 mRNA stabilization.

Zinc is known to induce multiple signalling events, particularly via the PI3K pathway [19, 21, 22, 34]. For example, zinc enhances gastrointestinal barrier function via activation of the PI3K/Akt signalling cascade in intestinal cells [34]. In this study zinc rapidly activated Akt phosphorylation. Inhibition of PI3K with LY294002 blocked zinc-induced iron absorption. These effects were not seen with inhibitors of the JNK or PKC pathways. While LY294002 can block numerous signalling pathways, it is a highly potent inhibitor of PI3K signalling and the inhibition of zinc-induced Akt phosphorylation and DMT1 expression by LY294002 implies a central role for PI3K in mediating these events. In agreement with the activation of a zinc-PI3K/Akt cascade, LY294002 also inhibited zinc-induced IRP2 protein expression. This is consistent with data from others demonstrating that insulin-induced stabilization of TfR1 mRNA in

HeLa cells is also mediated via a PI3K/IRP2-dependent mechanism [33]. In addition, zinc has been shown to have insulin-mimetic effects in activating PI3K/Akt signalling cascade in other cell systems [19]. Taken together these results suggest that a zinc-PI3K-IRP2 axis is essential for mediating DMT1 mRNA stability and promoting protein expression. In addition to inhibiting zinc induced DMT1 expression, LY294002 alone also down-regulated DMT1 (+IRE) mRNA levels. This implies that the PI3K pathway plays a critical role in maintaining basal DMT1 expression and intestinal iron absorption. Thus, targeting the PI3K pathway might serve as a therapeutic route to modulate intestinal iron absorption.

In summary, these results demonstrate that zinc stimulates intestinal iron absorption by induction of DMT1 expression via a PI3K/IRP2 dependent mechanism. This is the first demonstration that PI3K pathway is involved in regulating the intestinal iron absorption via modulation of IRP2 and could be potentially exploited to improve iron nutrition and metabolism. Given the likely co-existence of iron and zinc deficiencies in populations subsisting on phytic acid-rich vegetarian diets, consideration should be given to improving the zinc status to augment the efficacy of iron supplementation.

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Figure Legends

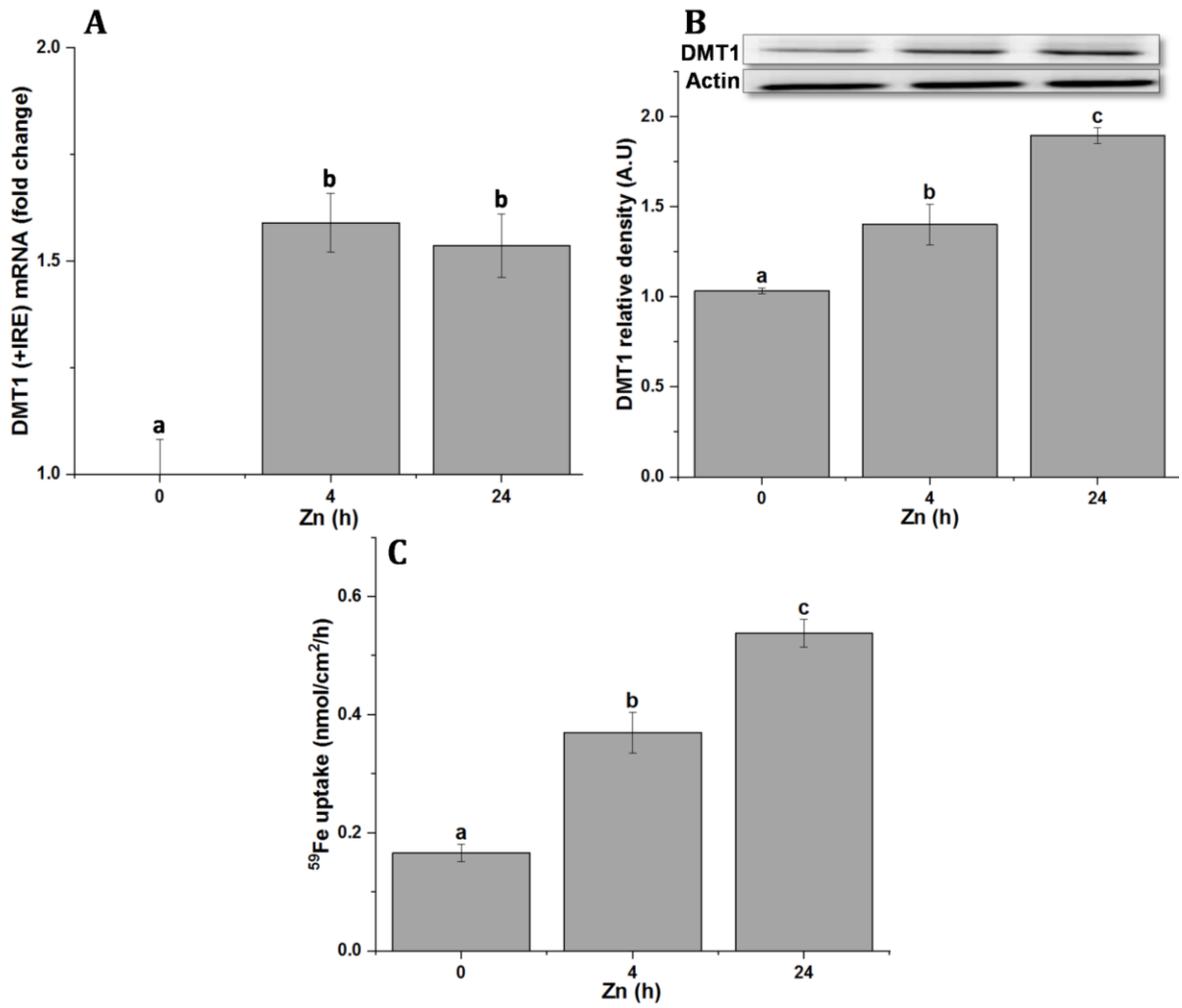

Figure 1. Effect of zinc on iron uptake and DMT1 expression in Caco-2 cells: Differentiated Caco-2 cells grown in 6-well plates were incubated with Zn (100 µmol/L) for indicated time. (A) ^{59}Fe uptake; (B) DMT (+IRE) mRNA expression; (C) DMT1 protein (~65kDa) expression. The iron uptake experiments were performed in triplicate and repeated twice to generate 6 independent observations. The qPCR was performed in triplicate and repeated thrice to generate 9 independent observations, and the data is normalized to the housekeeping gene, the β2-microglobulin. The immunoblots were repeated thrice, and the same blots were re-probed with β-actin (~45kDa). The densities were normalized to the respective housekeeping gene. The bars indicate the mean ±SEM and the bars that do not share common superscript differ significantly ($P<0.01$); Tukey's post-hoc test.

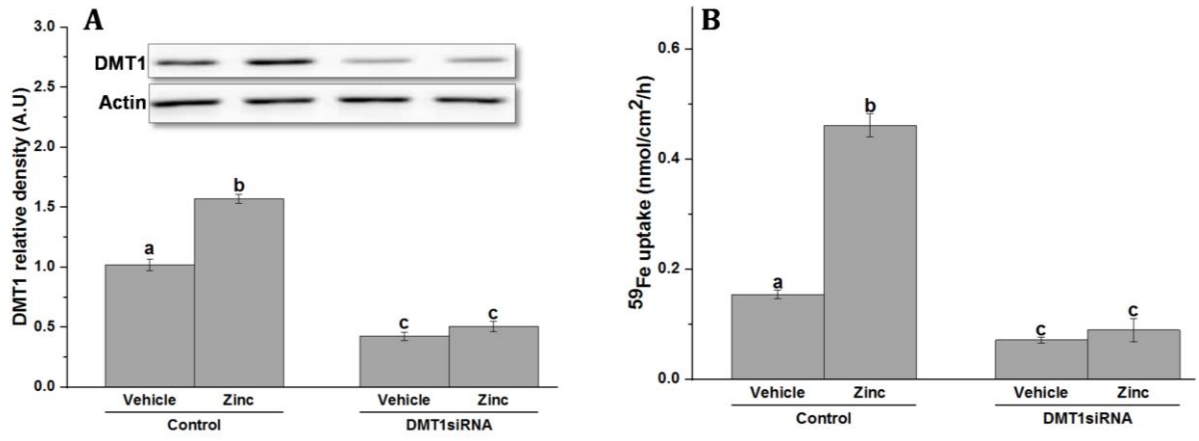

Figure 2. Effect of DMT1 silencing on zinc induced iron absorption in Caco-2 cells: Differentiated Caco-2 cells grown in 6-well plates either transfected with DMT1 siRNA (DMT1 siRNA group) or untransfected (control group) were incubated with vehicle (MEM) or Zn (100 µmol/L) for 24h. (A) DMT-1 protein (~65kDa); (B) ^{59}Fe uptake. The iron uptake experiments were performed in triplicate and repeated twice to generate 6 independent observations. The immunoblots were repeated thrice, and the same blots were re-probed with β-actin and densities were normalized. Two-way ANOVA found significant interaction between groups (control v DMT1siRNA; $P<0.01$) and treatment (± Zn; $P<0.01$). The bars indicate the mean ±SEM and the bars that do not share common superscript differ significantly ($P<0.001$); Tukey's post-hoc test.

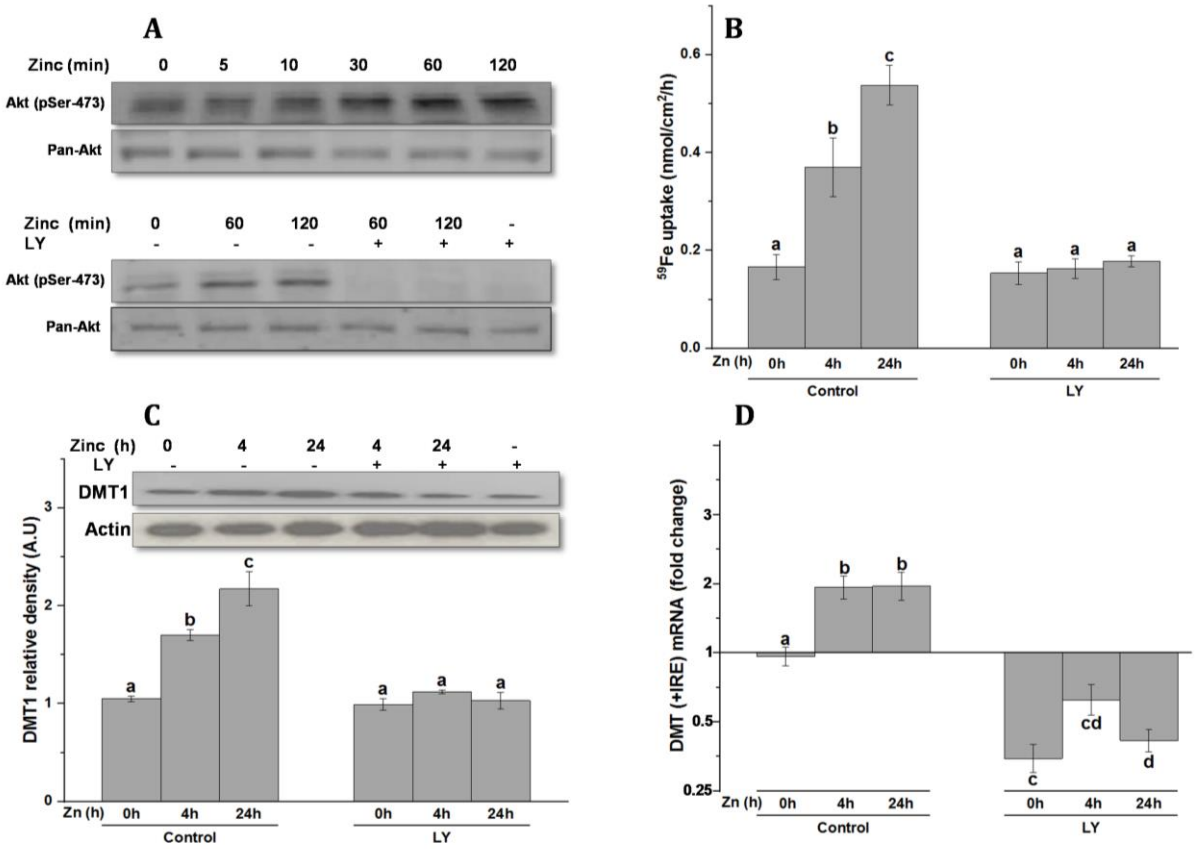

Figure 3. Effect of PI3K inhibitor on zinc induced iron absorption, DMT1, IRP2 expression: Differentiated Caco-2 cells grown in 6-well plates were incubated with Zn (100 µmol/L) for indicated times either in the absence (control group) or presence of LY 294002 (25 µmol/L; LY group). (A) Time course of Akt (p-Ser473; ~60 kDa) phosphorylation (top panel) and effect of LY294002 on zinc induced Akt phosphorylation (bottom panel) assessed by immunoblotting. (B) ^{59}Fe uptake (C) DMT1 (+IRE) mRNA expression (D) DMT1 protein (~65 kDa) expression. The immunoblots were repeated thrice, and the same blots were reported with pan Akt or β-actin as loading controls and the densities are normalized to the respective housekeeping protein. The iron uptake experiments were performed in triplicate and repeated twice to generate 6 independent observations. The qPCR was performed in triplicate and repeated thrice to generate 9 independent observations, and the data is normalized to the housekeeping gene, β2-microglobulin. Two-way ANOVA found significant interaction between groups (control v zinc; $P<0.01$) and treatment (± LY294002; $P<0.01$). For B and D there was also a significant interaction between group x treatment ($P<0.01$). The bars indicate the mean ± SEM and the bars that do not share common superscript differ significantly ($P<0.05$); Tukey's post-hoc test.

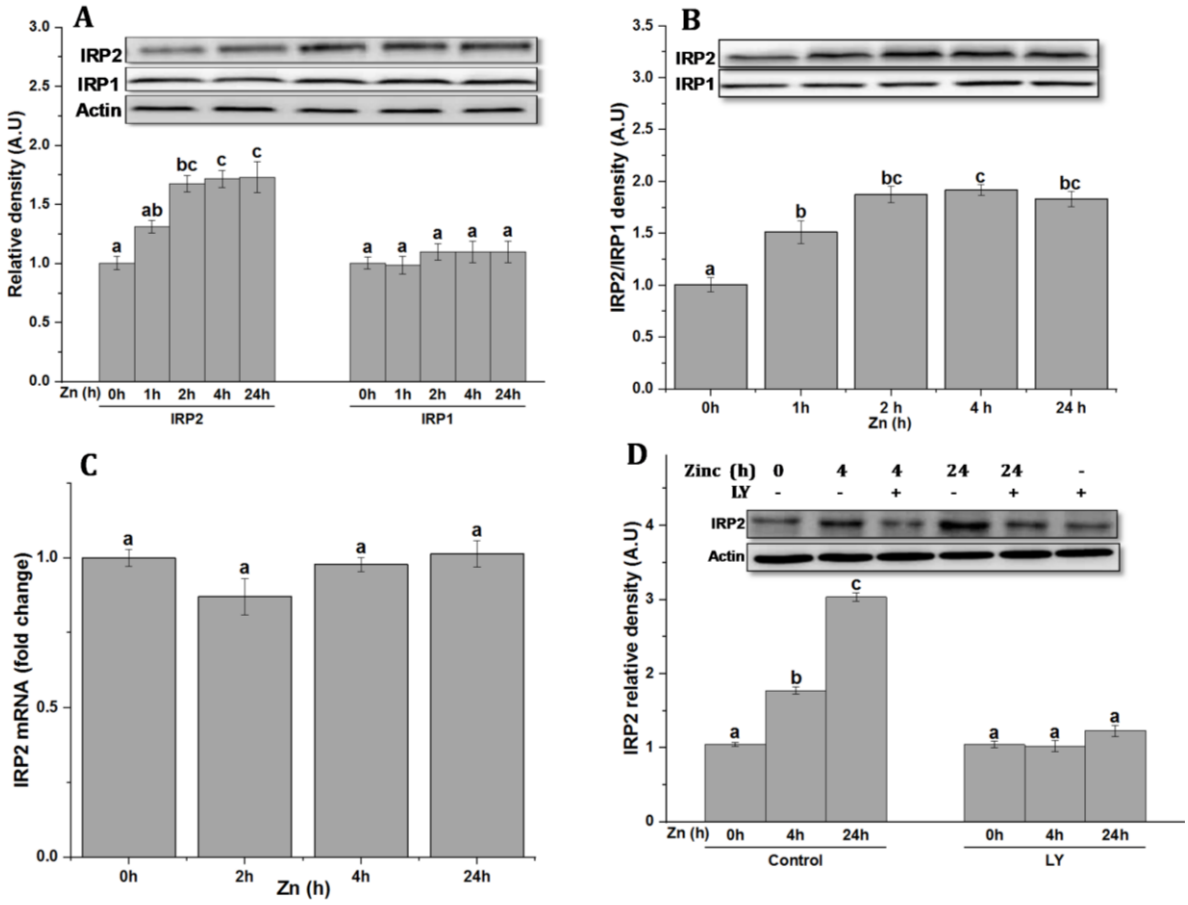

Figure 4. Effect of zinc and/or PI3K inhibitor on IRP1, IRP2 expression and activity: Differentiated Caco-2 cells grown in 6-well plates were incubated Zn (100 µmol/L) and/or LY294002 (25µmol/L) for indicated times. (A) immunoblot blot of IRP2 (~90kDa) and IRP1 (~90kDa) in total cell lysates (B) immunoblot of IRP2 and IRP1 levels in polysomal fraction (C) IRP2 mRNA (D) immunoblot of IRP2 in the presence and absence of zinc and/or LY294002.The qPCR was performed in triplicate and repeated thrice to generate 9 independent observations, and the data is normalized to the housekeeping gene, the β2-microglobulin. The immunoblots were repeated thrice, and the same blots were re-probed with β-actin. Data were analysed using either one-way ANOVA (A-C) or two-way ANOVA (D). Two-way ANOVA found significant interaction between groups (control v zinc; $P<0.01$) and treatment (± LY294002; $P<0.01$). There was also a significant interaction between group x treatment ($P<0.01$). The bars indicate the mean ±SEM and the bars that do not share common superscript differ significantly ($P<0.05$); Tukey's post-hoc test.

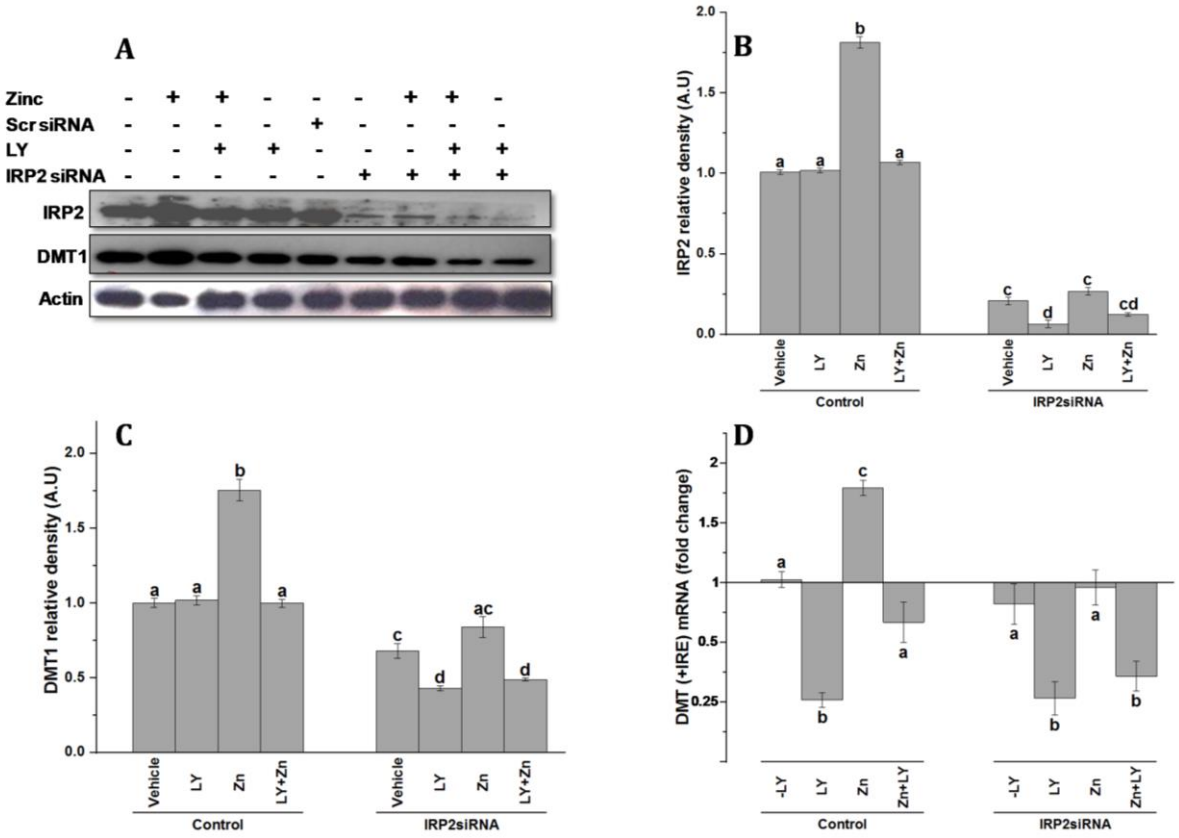

Figure 5. Effect of IRP2 siRNA silencing on zinc induced changes in DMT1 protein and mRNA expression: Differentiated Caco-2 cells grown in 12-well plates were transfected with IRP2 (IRP2 siRNA group) or control scrambled siRNA or untransfected (control group) followed by Zn (100 µmol/L) and/or LY294002 (25µmol/L) treatment for 24h. (A) IRP2 (~90kDa) and DMT-1 (~65kDa) immunoblots; (B) densities of IRP2 and (C) DMT1; (D) DMT1 (+IRE) mRNA expression. The immunoblots were repeated thrice, and the same blots were re-probed with β-actin. The qPCR was performed in triplicate and repeated thrice to generate 9 independent observations, and the data is normalized to the housekeeping gene, the β2-microglobulin. Two-way ANOVA found significant differences between groups (untransfected v IRP2siRNA; $P<0.01$) and treatment (Zn ± LY294002; $P<0.01$). There was also a significant interaction between group x treatment ($P<0.01$). The bars indicate the mean ± SEM and the bars that do not share common superscript differ significantly ($P<0.05$); Tukey's post-hoc test.

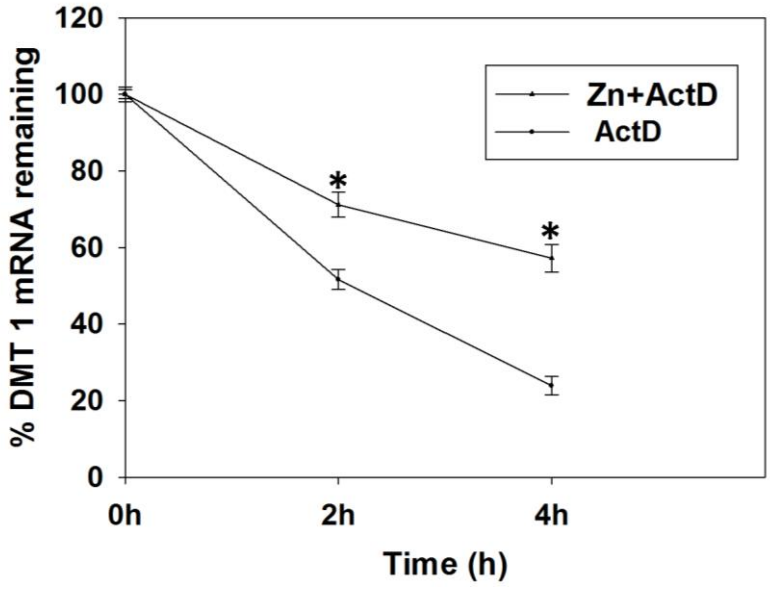

Figure 6. Effect of zinc on DMT1 mRNA stability: DMT1 mRNA levels in Caco-2 cells incubated either in the presence or absence of Zn and/or actinomycin-D (Act D; 10μg/mL) for 0, 2 and 4h time. The qPCR was performed in triplicate and repeated thrice to generate 9 independent observations, and the data is normalized to the housekeeping gene β2-microglobulin. Two-way ANOVA found significant interaction between groups (control vs Act D; $P<0.01$) and treatment (time; $P<0.01$). *$P<0.001$ compared to Act D at respective times; Tukey's post-hoc test.